Unveiling the secrets of the primary structure of Phl p 4

Molecular cloning of the major pollen allergen from Timothy Grass (*Phleum pratens*

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Introduction

Grass pollen allergy is one of the most common allergies worldwide. Recombinant allergens are believed to represent the future of allergen specific immunotherapy. Whereas the cDNA sequences of several grass pollen allergens are known, the coding sequence for Phl p 4, a major grass pollen allergen recognised by more than 70 % of allergic patients (1-5), has so far escaped detection (5).

Methods

A set of degenerate oligonucleotide primers was design based on N-terminal and internal protein sequences obtain from purified natural Phl p 4 (Tab. 1). In a complex PC strategy (Fig. 1) involving degenerate and specific prime the Phl p 4 gene could be amplified from genomic DNA a from cDNA derived from *Phleum pratense* pollen.

Results

The deduced amino acid sequence of full length Phl p 4 contains 500 amino acids, with a calculated MW of 55,7 kDa and a calculated basic pI of 8,8 (Tab. 2). The identity of the Phl p 4 sequence has been confirmed by positive reaction of recombinant Phl p 4 with specific monoclonal antibodies (Fig. 2) and by reaction with IgE from grass pollen allergics (Fig. 3). A sequence database homology search revealed similarities to a group of berberine bridge enzyme-like oxido-reductases (Fig. 4).

Tab. 2 Phl p 4 Sequence analysis

Amino acid		Number	% by weight	% by frequency
A	Ala	40	5.51	8.00
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Sequence analysis	Calculated values
Molecular weight	55,754 Da
Length	500 amino acids
Isoelectric point	8.80
N-Glycosylation sites	4
FAD-binding consensus sequence	Yes *

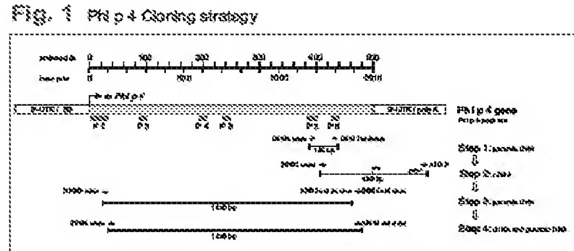
* To date the binding of a flavin co-factor could not be proved for purified natural or recombinant Phl p 4.

Tab. 1 N-terminal and internal peptide sequences of Phl p 4

Peptide	Sequence	From	To	Fragment
P 1a	[illegible]	1	33	N-terminal
P 1b	[illegible]	1	30	N-terminal
P 1c	[illegible]	1	10	N-terminal
P 1d	[illegible]	1	10	N-terminal
P 1e	[illegible]	1	10	N-terminal
P 1f	[illegible]	1	11	N-terminal
P 2	[illegible]	394	401	Glu-C
P 3	[illegible]	50	55	Glu-C
P 4	[illegible]	194	209	Lys-C
P 5	[illegible]	205	240	Lys-C
P 6	[illegible]	425	439	CNBr

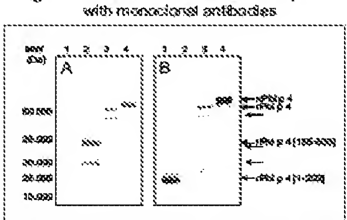
N-terminal sequencing of purified natural Phl p 4 and of fragments obtained from protease digestion or CNBr cleavage revealed the peptide sequences P1-P6. P1 a-f presumably represent different isoforms of natural Phl p 4.

Fig. 1 Phl p 4 Cloning strategy



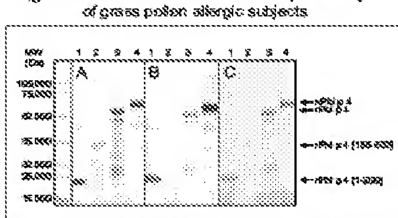
Step 1: Degenerate oligonucleotide primer pools (DP810 and DP837) based on the Phl p 4 peptide sequences P2 and P6 have been used in a PCR reaction to amplify a small 145 bp internal DNA fragment of genomic *Phleum pratense* DNA.
Step 2: Based on that sequence a specific oligonucleotide primer (SP852) was designed to be used in a 3' RACE PCR approach in combination with the anchor primer AUAP (Life technologies). A 425 bp fragment spanning the entire 3'-end of the Phl p 4 gene could be obtained.
Step 3: Two specific antisense primers (SP855 and SP856) have been designed on the basis of that sequence. These were used in combination with a degenerate sense primer pool (DP829) based on the N-terminal peptide P1 to amplify genomic Phl p 4 DNA in a "sense side mixed oligonucleotide, antisense side nested unique primer" PCR approach. A resulting 1320 bp fragment has been cloned and sequenced. The sequences of the peptides P2-P6 could be aligned with the deduced amino acid sequence of this fragment confirming the identity with the Phl p 4 gene.
Step 4: A specific sense primer (SP868) has been designed and was used together with SP856 antisense to amplify a 1336 bp fragment of Phl p 4. Several independent PCR-products from genomic DNA as well as from cDNA have been sequenced to exclude PCR errors. Different variants of Phl p 4 could be detected. The identity of cDNA and genomic clones show that no intron sequences are present in the amplified region.

Fig. 2 Reaction of recombinant Phl p 4 with monoclonal antibodies



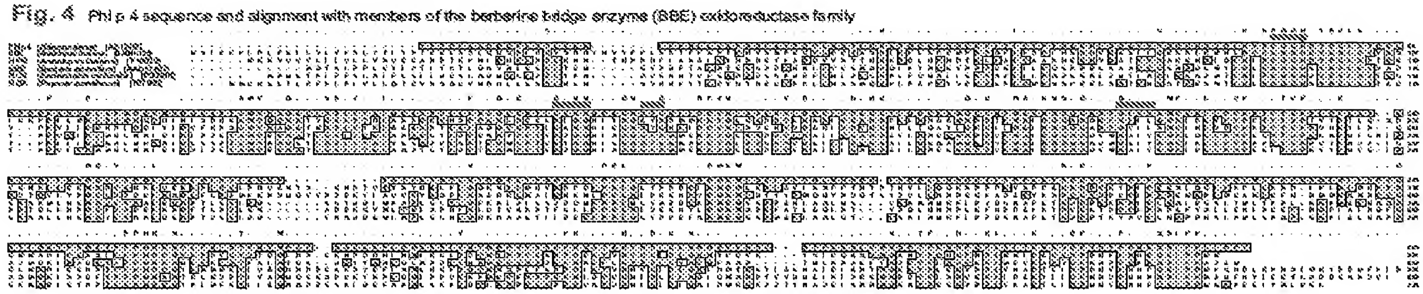
Western blot of whole cell extracts of E. coli expressing (1) a N-terminal fragment of Phl p 4 (aa 1-200, MW ≈ 22 kDa), (2) a C-terminal fragment of Phl p 4 (aa 185-500, MW ≈ 36 kDa), (3) full length recombinant Phl p 4, and (4) purified natural Phl p 4.
A: The monoclonal antibody 5H1 detects nPhl p 4, rPhl p 4 and the C-terminal fragment. B: The monoclonal antibody 3C4 detects nPhl p 4, rPhl p 4 and the N-terminal fragment of Phl p 4.

Fig. 3 Reaction of recombinant Phl p 4 with IgE of grass pollen allergic subjects



Western blot of whole cell extracts of E. coli expressing (1) a N-terminal fragment of Phl p 4 (aa 1-200, MW ≈ 22 kDa), (2) a C-terminal fragment of Phl p 4 (aa 185-500, MW ≈ 36 kDa), (3) full length recombinant Phl p 4, and (4) purified natural Phl p 4. Incubation with sera of three different grass pollen allergic individuals (A, B, C) confirmed the IgE reactivity of recombinant Phl p 4.

Fig. 4 Phl p 4 sequence and alignment with members of the berberine bridge enzyme (BBE) oxidoreductase family



Multiple alignment of Phl p 4, BBE (berberine bridge enzymes), HPR (hypothetical protein) and RDX (reticuline oxidase) sequences. Residues identical to the Phl p 4 sequence are shown in yellow. Phl p 4 shows 34 % identical amino acid residues with BBE from E. californica and 43 % with BBE from A. thaliana. The oxidoreductase family flavin-binding consensus sequences are marked with blue lines, the conserved histidine to which the FAD is covalently bound in BBE is marked in red [illegible].

Conclusion

The ability to produce recombinant Phl p 4, a major allergen of grass pollen with one of the highest IgE binding frequencies measured in sera of pollen allergic patients, may represent a key step for the development of future diagnostic and immunotherapeutic preparations.

References

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